# Polarized Th1 and Th2 cells are less responsive to negative feedback by receptors coupled to the AC/cAMP system compared to freshly isolated T cells

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# Polarized Th1 and Th2 cells are less responsive to negative feedback by receptors coupled to the AC/cAMP system compared to freshly isolated T cells

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**1** The adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP) system is known to negatively regulate transcriptional activity of T cells, thereby possibly modulating T-cell-mediated responses at the sites of inflammation. Effects of cAMP have been widely studied in freshly isolated T cells and T-cell clones; yet, effects in differentiated Th1 and Th2 cells are largely unknown.

**2** To obtain differentiated T helper cells, we activated naive T cells for 1 week in the presence of IL-12 plus $\alpha$-IL-4 to generate Th1-type cells and in the presence of IL-4 plus $\alpha$-IL-12 to generate Th2-type cells.

**3** We demonstrate that, in contrast to freshly isolated T cells, the production of Th1 (IFN-$\gamma$) and Th2 (IL-4, IL-5) cytokines in polarized T helper cells is not strictly controlled by the activation of AC/cAMP-linked $\beta_2$-adrenergic and prostaglandin (PG)$E_2$ receptors.

**4** In Th2 cells, $PGE_2$ could still activate the $G_s$ protein-coupled AC/cAMP system and subsequently induce CREB phosphorylation, whereas $PGE_2$ was unable to activate the cAMP-dependent pathway in Th1 cells. In both Th1 and Th2 cells, the induction of CREB phosphorylation by $\beta_2$-agonist fenoterol was impaired.

**5** The loss of control over cytokine production by cAMP elevating agents in differentiated Th1 and Th2 subsets may have important implications for the regulation of Th1- and Th2-mediated diseases, in particular those associated with the ongoing immune responses.

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**Keywords:** Th1; Th2; cytokines; $\beta_2$-adrenergic; $PGE_2$; cAMP; CREB

**Abbreviations:** AC, adenylyl cyclase; $\beta_2$AR, $\beta_2$-adrenergic receptor; $\beta$ARK, $\beta$-adrenergic receptor kinase; $\beta_2\mu$G, $\beta_2$-microglobulin; cAMP, cyclic adenosine monophosphate; CRE, cAMP responsive element; CREB, cAMP-responsive element binding protein; db, dibutyryl; GRK, G protein-coupled receptor kinase; IBMX, 1-methyl-3-isobutylxantine; JNK, c-Jun N-terminal kinase; MAP, mitogen activated protein; PDE, phosphodiesterase; $PGE_2$, prostaglandin $E_2$; PI3-kinase, phosphatidylinositol 3-kinase; PKA, protein kinase A; PKC, protein kinase C; Th, T helper

## Introduction

The cyclic adenosine monophosphate (cAMP)-dependent pathway is an important negative feedback system in the regulation of inflammatory activity. Expression of cytokine production in T lymphocytes is regulated by prostaglandin (PG)$E_2$ and $\beta_2$-agonists, which activate receptors coupled to the cAMP-dependent pathway. Upon receptor binding, the associated $G_s$ protein triggers adenylyl cyclase (AC) activity, resulting in the formation of intracellular cAMP. Cytokine genes containing a cAMP-responsive element (CRE) in their promoter, like the IFN-$\gamma$ gene, can be regulated by the protein kinase A (PKA)-mediated phosphorylation of cAMP-responsive element binding protein (CREB) (Nigg *et al*., 1985; Gonzalez & Montminy, 1989; Masquilier & Sassone-Corsi, 1992). Not all cytokine genes that are controlled by the cAMP-dependent pathway contain a CRE in their promoter. For instance, no binding site for CREB has been found in the IL-5 promoter region. Expression of cytokine genes lacking this binding site can be affected indirectly by cAMP, most likely by the modulation of the signal transduction pathways, including the mitogen activated protein (MAP) kinase pathways (Wu *et al*., 1993; Tamir *et al*., 1996; Harada *et al*., 1999).

In T cells, cAMP elevating substances are known to dose-dependently control the production of both Th1 and Th2 cytokines (Betz & Fox, 1991; Snijdewint *et al*., 1993; Hilkens *et al*., 1995; Borger *et al*., 1996, 1998, 1999), although the ultimate effect on Th2 cytokines appears to be dependent on the activation state and costimulatory signals (Hilkens *et al*., 1995; Borger *et al*., 1996, 1998). cAMP has been described to

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inhibit Th2 cytokine production in freshly isolated T cells activated with CD3 plus CD28 antibodies (Borger *et al.*, 1996), whereas it slightly enhances the production of IL-5 in the presence of potent PKC activators or high concentrations of IL-2 (Borger *et al.*, 1996, 1998) and strongly upregulates IL-5 in Th2 clones (Snijdewint *et al.*, 1993; Lee *et al.*, 1994). Since T-cell clones may be dysregulated because of many cell divisions, this effect in Th2 clones may not be representative for *in vivo* conditions. Furthermore, the existence of strictly separated Th1 and Th2 subsets in the human immune system has been under discussion. However, polarized Th1 and Th2 cells may exist at the sites of tissue inflammation; several immune responses are associated with the presence of Th1 and Th2 subsets, including Th1-mediated autoimmune diseases and Th2-mediated allergies (Abbas *et al.*, 1996; O'Garra, 1998). At the initiation of an immune response, autocrine IL-2 induces the proliferation of naive helper cells. Subsequently, the specific cytokine environment determines the final outcome of the T helper subtype (Abbas *et al.*, 1996; O'Garra *et al.*, 1998). From *in vitro* studies, it has become clear that naive $CD4^+$ cells differentiate into polarized Th1 and Th2 during activation in the appropriate environment, for example, in the presence of IL-12 (Hsieh *et al.*, 1993; Seder *et al.*, 1993) or IL-4 (Seder *et al.*, 1992), respectively. In addition, when T cells are only activated for a short period or in the absence of specific cytokine environment, nonpolarized intermediates or Th0 cells develop, which are able to produce various patterns of cytokines. Recent studies have demonstrated that entering the cell cycle is essential to induce epigenetic remodeling, that is, alterations in chromatin structure, which is crucial for differentiation towards a Th1 or Th2 phenotype and the efficient production of the associated cytokines (Argawal & Rao, 1998; Bird *et al.*, 1998). The effects of cAMP have not been examined thoroughly in polarized Th1 and Th2 subsets, although this may provide insight into the regulation of Th1- and Th2-associated immune responses. We investigated the regulation of cytokine production by cAMP in Th1 and Th2 cells and demonstrate that control over cytokine production by cAMP elevating agents operating via AC-coupled receptors is partially lost in Th2 subsets and completely lost in Th1 subsets.

## Experimental procedures

### *Isolation of T cells*

Peripheral blood cells were obtained from healthy volunteer platelet donors. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll – Hypaque (Lymphoprep; Nycomed, Oslo, Norway) density-gradient centrifugation. T cells were isolated by rosetting with 2-aminoethylisothioronium bromide (AET)-treated sheep red blood cells (SRBC). The SRBC were lysed with 155 mmol l$^{-1}$ $NH_4Cl$, 10 mmol l$^{-1}$ $KHCO_3$ and 0.1 mmol l$^{-1}$ EDTA. After isolation, T cells were incubated overnight at 37°C in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 5% fetal calf serum (FCS; Hyclone, Logan, UT, U.S.A.), supplemented with 100 U ml$^{-1}$ penicillin and 100 $\mu$g ml$^{-1}$ streptomycin.

### *Th1/Th2 polarization*

T lymphocytes were isolated from peripheral blood of healthy volunteer platelet donors as described above. The T cells were differentiated into Th1 and Th2 subsets as described before (Roozendaal *et al.*, 2001). In short, naive helper T lymphocytes were sorted after staining with α-CD45RO-FITC (UHCL-1) and α-CD4 CyQ (B-F5) (Immune Quality Products (IQP), Groningen, The Netherlands using a MoFlow™ Flow cytometer (Cytomation, Fort Collins, CO, U.S.A.) calibrated using Flow-Check™ Fluorospheres (Beckman Coulter, Paris, France). Purity was above 98% by reanalysis. Cells were cultured in RPMI 1640 medium containing 10% FCS, in the presence of PHA, IL-2, irradiated allogenic PBMC (neutral conditions) and either IL-12 (2 ng ml$^{-1}$, R&D systems, ITK diagnostics, Uithoorn, The Netherlands) plus α-IL-4 (200 ng ml$^{-1}$, Becton Dickinson, Erebodegem-Aalst, Belgium) to generate polarized Th1 cells, or IL-4 (200 U ml$^{-1}$, Becton Dickinson) and α-IL-12 (2 $\mu$g ml$^{-1}$, R&D systems) to generate polarized Th2 cells. To confirm that Th1 and Th2 phenotypes were obtained after 7 days, the cells were analyzed for intracellular cytokines as described before (Jung *et al.*, 1993). In short, the polarized T helper cells were replated in RPMI medium containing 5% FCS and cultured overnight. Next, the cells were stimulated with PMA (10 ng ml$^{-1}$) and ionomycin (1 $\mu$g ml$^{-1}$) for 4 h in the presence of monensin (2 $\mu$M, Alexis, Läufelfingen, Switzerland). Cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% saponin/0.1% azide and stained using α-CD4-CyQ (B-F5, IQP, Groningen), α -IFN-γ-FITC (45-15, IQ P) and α-IL-4-PE (B-T4, CLB, Amsterdam, The Netherlands). Irrelevant specificity antibodies of the same isotype were used for gate setting. Analysis was performed using an Elite™ flow cytometer (Beckman Coulter). Lymphocyte events were gated on the basis of forward and sideward scatter characteristics. The intracellular cytokine stainings indicated that highly divergent cytokine production patterns were obtained after 1 week of culture. Virtually all cells became $CD45RO^+$ after culturing under polarizing conditions. In the cell population polarized under Th1 conditions, approximately 40% of the $CD4^+$ cells was IFN-$\gamma^+$/IL-$4^-$ and 0.5% was IL-$4^+$/IFN-$\gamma^-$, whereas in the cell population cultured under Th2 conditions, approximately 10% was IL-$4^+$/IFN-$\gamma^-$ and 0.5% was IL-$4^-$/IFN-$\gamma^+$. In both cell populations, only a small percentage of the cells was positive for both cytokines.

### *Stimulation of the T cells*

After 7 days of culture under polarizing conditions, the cells were rested overnight in RPMI medium containing 5% FCS. For stimulation, polarized T helper cells or freshly isolated T cells ($1 - 3 \times 10^6$ ml$^{-1}$) were incubated in RPMI 1640 medium containing 5% FCS with 50 $\mu$l ml$^{-1}$ of α-CD3 and α-CD28 antibodies, as previously described by Borger *et al.* (1999), in the presence or absence of $PGE_2$ (Sigma, St Louis, MO, U.S.A.) in a final concentration of 10 ($\mu$M $\beta_2$-agonist fenoterol (Sigma) in a final concentration of 10 $\mu$M, cAMP analog dibutyryl (db)-cAMP (Boehringer-Mannheim GmbH, Germany) in a final concentration of 0.5 $\mu$M or phosphodiesterase (PDE) inhibitor l-methyl-3-isobutylxantine (IBMX, Alexis, Läufelfingen, Switzerland) in a final concentration of 100 $\mu$M.

### *Measurement of cytokine protein*

Polarized T cells or freshly isolated T cells ($1 - 3 \times 10^6$ ml$^{-1}$) were left unstimulated or stimulated with α-CD3/α-CD28 during 6 – 8 h. Secreted IL-4, IL-5 and IFN-γ proteins were measured in cell-free supernatants, using enzyme-linked immunosorbent assay (ELISA) kits for IL-4 and IFN-$\gamma$ (CLB). The IL-5 ELISA was performed as previously described by Hoekstra *et al.* (1997).

### *Measurement of intracellular cAMP accumulation*

After resting overnight, T lymphocytes ($3 \times 10^6\,ml^{-1}$) were suspended in RPMI 1640 medium. Stimulation of cAMP production was performed as described before (Meurs *et al.*, 1980). In short, the samples were incubated with IBMX (0.5 mM) for 10 min to prevent cAMP degradation. After preincubation, the samples were stimulated for 10 min with $PGE_2$ (10 $\mu$M). Reactions were terminated by adding 2 N HCl – 0.1 M EDTA followed by incubating the samples at 80°C for 10 min. After centrifugation of precipitated protein, the samples were neutralized by $CaCO_3$ and cAMP was measured using an enzyme immunoassay (Biotrak, Amersham, Buckinghamshire, U.K.) according to the manufacturer's guidelines. cAMP concentrations are expressed as fmol cAMP/$10^6$ T lymphocytes.

### *Immunodetection by Western blotting*

Phosphorylation of CREB and expression of G protein-coupled receptor kinase 3 (GRK3/$\beta$ARK2) were analyzed by Western blotting. T cells ($1-3 \times 10^6$) were cultured overnight in 1 1/2 ml RPMI 1640 medium containing 0.5% FCS. The cells were incubated with $PGE_2$ (10 $\mu$M), fenoterol (10 $\mu$M), db-cAMP (0.5 mM), IBMX (100 $\mu$M) or sodium fluoride (NaF, Sigma, 10 mM), to directly activate the G protein, in final a concentration of 10 mM for 60 min to study CREB phosphorylation, or left unstimulated to study $\beta$ARK expression. T cells were harvested and spun down at maximum speed during 30 s. Next, total cell lysates were obtained by resuspension of the pellets in 1 × sample buffer (containing 2% SDS, 10% glycerol, 2% $\beta$-mercaptoethanol, 60 mM Tris-Cl pH 6.8 and bromophenol blue) and boiling for 5 min. Samples were loaded on a SDS 10% PAGE gel (acrylamide : bisacrylamide 173 : 1) and transferred to a cellulosenitrate membrane (Schleicher & Schuell, Germany). Immunoblotting was performed by standard procedures and the detection was performed according the manufacturer's guidelines (ECL, Amersham). Relative protein levels were quantified using the gelscan program Diversity One (Pharmacia, Uppsala, Sweden).

### *Reverse transcription (RT) – polymerase chain reaction (PCR)*

Polarized T cells ($5 \times 10^6$) were rested overnight in RPMI medium containing 5% FCS, harvested and RNA was isolated using the TRIzol method (GIBCOBRL, Burlington, Ontario, Canada). Total cellular RNA was resuspended in diethyl-pyrocarbonate (DEPC; Sigma) treated $H_2O$. A volume of 1 $\mu$g RNA was used for cDNA synthesis. First, the samples were incubated during 10 min at 65°C with a random hexamer (pdN6). After cooling on ice, RT mix containing 5 × RT buffer (GIBCOBRL), 0.1 M DTT, 5 mM of each dNTP and 3 U of Reverse Transcriptase (GIBCOBRL) was added and the samples were incubated at 37°C for 1 h. For the PCR reaction, 10 × PCR buffer (GIBCOBRL), 50 $\mu$M of forward and reverse primer, 0.25 $\mu$l *Taq* polymerase, 2 mM dNTP's and 75 $\mu$l $MgCl_2$ in 25 $\mu$l total volume were added. The following specific primer pairs for $\beta_2$ microglobuline ($\beta_2\mu$G, housekeeping gene), $EP_2$ (an AC-coupled subtype of the $PGE_2$ receptor), EP3 (a $G_i$-coupled subtype of the $PGE_2$ receptor) and $\beta_2$-adrenergic receptor ($\beta_2$AR) were obtained from Biolegio BV (Malden, The Netherlands):

$\beta_2\mu$G: 5′CCAGCAGAGAATGGAAAGTC3′ sense and 5′GATGCTGCTTACATGTC TCG3′ antisense. $\beta_2$AR: 5′CC TTCTTGCTGGCACCCCAT3′ sense and 5′GGAAGTCCA AAACTCGCACCA3′ antisense. $EP_2$: 5′CCTCCTGAGAAA GACAGTGCT3′ sense and 5′AAGACACTCTCTGAGTC CT3 antisense. $EP_3$: 5′TGCTGGGCGTGGGCCGCTACA3′ sense and 5′GACCAACAGACGGACAGCACA3′ antisense. PCR conditions were a denaturation step at 94°C for 5 min followed by 20 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for detection of $\beta_2\mu$G, 35 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 30 s for detection of $\beta_2$AR, 25 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for detection of $EP_2$ and 30 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 30 s for detection of $EP_3$. With these primers, the amplified products were 268, 295, 395 and 300 bp long for $\beta_2\mu$G, $\beta_2$AR, $EP_2$ and EP3, respectively. After PCR, 10 $\mu$l of the reaction mixture was run on a 1.5% agarose gel containing 0.2 $\mu$g ethidium bromide in 1 × TAE buffer. A 100 bp ladder (Pharmacia) was used as DNA marker. Relative mRNA levels were quantified using the gelscan program Diversity One (Pharmacia, Uppsala, Sweden).

**Table 1** Absolute amounts of secreted cytokine protein in freshly isolated and polarized T cells

| *T cell type* | *Treatment* | *Cytokine secretion in pg ml$^{-1}$* | | |
|---|---|---|---|---|
| | | *IFN-$\gamma$* | *IL-4* | *IL-5* |
| Freshly isolated | Basal | ND | ND | ND |
| | α-CD3/α-CD28 | 3539 ± 3037 | 15.3 ± 12.8 | 54.8 ± 47.5 |
| | + PGE2 10 $\mu$m | 33.6 ± 39.2 | 5.3 ± 3.8 | 4.5 ± 3.1 |
| | + fenoterol 10 $\mu$m | 679 ± 310 | 10.1 ± 9.3 | 26.5 ± 23.9 |
| Polarized | Basal | 455 ± 347 | 0.4 ± 0.1 | 14.2 ± 15.8 |
| | α-CD3/α-CD28 | 10213 ± 7803 | 84.4 ± 88.8 | 506 ± 316 |
| | + PGE2 10 $\mu$m | 10018 ± 4050 | 58.3 ± 37.8 | 400 ± 241 |
| | + fenoterol 10 $\mu$m | 10200 ± 4020 | 79.4 ± 42.5 | 499 ± 385 |

Freshly isolated T cells or polarized Th cells were left unstimulated or stimulated by α-CD3/α-CD28 in the presence and absence of 10 $\mu$M $PGE_2$ or fenoterol for 6 – 8 h IFN-$\gamma$ protein was measured in supernatant from freshly isolated T cells and polarized Th1 cells. IL-4 and IL-5 proteins were measured in supernatant from freshly isolated T cells and polarized Th2 cells. Results arc expressed as means ± s.e.m., $n = 8$. ND: not detectable.

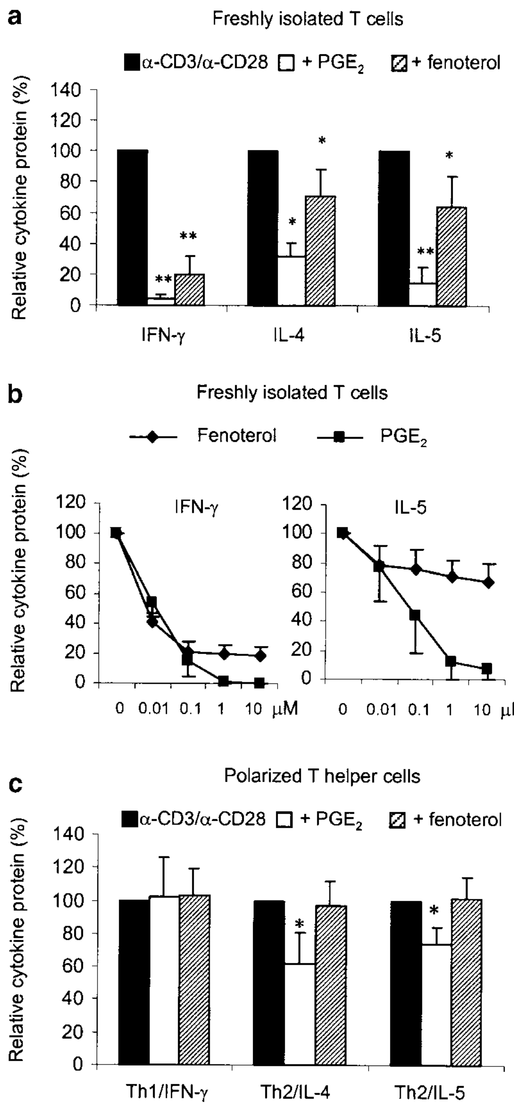


**Figure 1** (a) Th1 and Th2-like cytokine production is significantly inhibited by cAMP elevating agents in freshly isolated T cells. T lymphocytes were isolated from healthy donors and rested overnight. $PGE_2$ and fenoterol were added in a concentration of 10 $\mu$M and cells were subsequently stimulated with α-CD3/α-CD28 for 8 h. IFN-γ, IL-4 and IL-5 protein secretion were measured in cell-free supernatants. Protein levels are expressed as percentage ($x \pm$s.e.m., $n=8$) of the secretion after stimulation with α-CD3/α-CD28 in the absence of cAMP elevating agents. $^{*}P<0.05$ and $^{**}P<0.001$ for the cytokine secretion levels after preincubation with cAMP elevating agents compared to the level without these agents. (b) Dose–response curves for $PGE_2$ and fenoterol on the secretion of IFN-γ and IL-5. Freshly isolated T cells were incubated with different concentrations of $PGE_2$ or fenoterol and subsequently stimulated with α-CD3/α-CD28 for 8 h. IFN-γ, IL-4 and IL-5 protein secretions were measured in cell-free supernatants. Protein levels are expressed as percentage ($x \pm$s.e.m., $n=4$) of the secretion after stimulation with α-CD3/α-CD28 in absence of cAMP elevating agents. (c) Cytokine production in polarized Th1 and Th2 cells is not under strict control of cAMP elevating agents. Polarized Th1 and Th2 cells were rested overnight in RPMI medium containing 5% FCS and stimulated for 6 h with α-CD3/α-CD28. $PGE_2$ and fenoterol were added prior to stimulation, in a concentration of 10 $\mu$M. IFN-γ, IL-4 and IL-5 proteins were measured in supernatants. Protein levels are expressed as percentage ($x \pm$s.e.m., $n=8$) of the secretion after stimulation with α-CD3/α-CD28 in the absence of cAMP elevating agents. $^{*}P<0.05$ for the cytokine secretion levels after preincubation with cAMP elevating agents compared to the level without these agents.

*Statistical analysis*

For the protein measurements, statistical analysis was performed using a nonparametric test for paired observations (Wilcoxon-signed ranks test). Statistical significance of the secretion data was set at $P<0.05$.

# Results

*Control over cytokine production by cAMP elevating agents in polarized helper T cells is reduced compared to freshly isolated T cells*

There was a wide range in the levels of cytokines secreted by the different blood donors. Absolute values (mean $\pm$ s.e.m.) of cytokine secretion are given in Table 1. Since similar effects were exerted by cAMP elevating agents in low and high cytokine producers, cytokine protein levels are expressed as a percentage of the cytokine secretion upon stimulation in the absence of the cAMP elevating agents. As demonstrated in Figure 1a, secretion of IFN-γ was strongly inhibited by 10 $\mu$M $PGE_2$ (from 100 to $4 \pm 3\%$, $P<0.001$, $n=8$) and to a smaller extent by 10 $\mu$M of short-acting $\beta_2$-agonist fenoterol (from 100 to $20 \pm 12\%$, $P<0.001$, $n=8$) in freshly isolated, α-CD3/α-CD28 stimulated T cells. The secretion of the Th2 cytokines IL-4 and IL-5 was also under firm control of 10 $\mu$M $PGE_2$ in these cells (inhibition from 100 to $32 \pm 9$ and $15 \pm 10\%$, respectively, $P<0.001$). Fenoterol of 10 $\mu$M moderately, but significantly, reduced IL-4 and IL-5 secretion (from 100 to $71 \pm 17$ and $64 \pm 20\%$, respectively, $P<0.03$, Figure 1a). Dose-dependent effects of fenoterol and $PGE_2$ on cytokine production in stimulated freshly isolated T cells are depicted in Figure 1b ($n=4$). The secretion of IFN-γ appeared to be more sensitive to fenoterol and $PGE_2$ compared to the secretion of IL-5, whereas IFN-γ production was still significantly inhibited by 10 nm fenoterol and $PGE_2$ ($P<0.01$). IL-5 was only significantly inhibited when fenoterol and $PGE_2$ were used in higher concentrations (from 100 nm to 10 $\mu$M).

Interestingly, cytokine production appeared to be differently regulated by cAMP elevating agents in polarized T helper cells. In contrast to the strong inhibitory effects on IFN-γ protein secretion in freshly isolated T cells (Figure 1a), there was a complete absence of control over IFN-γ secretion by $PGE_2$ and fenoterol (10 $\mu$M) in polarized Th1 cells (Figure 1b). In polarized Th2 cells, the control of IL-4 and IL-5 secretion was also reduced compared to freshly isolated T cells. No inhibitory effect was found upon stimulation of the $\beta_2$-adrenoceptor with fenoterol (10 $\mu$M). In contrast, the secretion of IL-4 and IL-5 protein was still modestly, but significantly, inhibited by 10 $\mu$M $PGE_2$ (from 100 to $62 \pm 19$ and $74 \pm 10\%$, $P<0.05$ and $P<0.01$, respectively). The loss of control in Th2 and Th1 subsets may be because of differential regulation of the cytokine gene promoters or by impaired activation of the cAMP-dependent pathway.

*CREB phosphorylation induced by different cAMP elevating agents*

To investigate whether the cAMP-dependent pathway can still be efficiently activated in polarized Th1 cells and Th2 cells, we studied the phosphorylation of downstream effector CREB. In freshly isolated T cells, both $PGE_2$ and fenoterol (10 $\mu$M) clearly induced CREB phosphorylation (Figure 2), the effect of $PGE_2$ being most pronounced. In contrast, in Th1 cells, no phosphorylation of CREB could be observed upon stimulation with $PGE_2$ and fenoterol. These results suggest that the defective regulation of cytokine production by fenoterol and $PGE_2$ in Th1 cells is because of impaired activation of the cAMP downstream pathway. In Th2 cells, $PGE_2$ was able to induce a clear increase in CREB phosphorylation, whereas fenoterol only slightly enhanced the levels of phosphorylated CREB. Thus, the ability of $PGE_2$ to activate the cAMP-dependent pathway is impaired in Th1 cells and still intact in Th2 cells, whereas $\beta_2$-adrenergic activation of this pathway appears to be disturbed in both Th1 and Th2 cells. To study if this $\beta_2$-adrenergic hyporesponsiveness in polarized cells is a consequence of the proliferation and activation induced by culturing under polarizing conditions, we studied the effect of neutral (Th0) conditions, that is, PHA, IL-2 and irradiated APC. In T cells cultured under these conditions for 7 days, fenoterol was not able to induce phosphorylation of CREB either. In contrast, $PGE_2$ could still enhance CREB phosphorylation. These data suggest that $\beta_2$-adrenergic hyporesponsiveness may develop by polyclonal activation of T cells during polarization.

### *Impaired formation of intracellular cAMP by $PGE_2$ in Th1 cells*

To study in more detail the defects of $PGE_2$ and $\beta_2$-receptor function, we measured the capacity of these agents to enhance the accumulation of intracellular cAMP in freshly isolated and polarized T cells. To prevent degradation of cAMP during the assay, cells were incubated with PDE inhibitor IBMX. Addition of IBMX strongly enhanced accumulation of intracellular cAMP in both polarized and freshly isolated T cells: from $0.32 \pm 0.06$ to $2.09 \pm 0.58$ pmol cAMP in Th1 cells, from $0.18 \pm 0.07$ to $1.83 \pm 1.0$ pmol cAMP in Th2 cells and from $0.22 \pm 0.09$ to $1.93 \pm 0.58$ pmol cAMP in freshly isolated T cells (Figure 3a). In both Th1 and Th2 cells, fenoterol was unable to increase intracellular cAMP levels (data not shown). Addition of $PGE_2$ (10 $\mu$M) induced a significant increase in cAMP production in both freshly isolated T cells and polarized Th2 cells (fold increase $263 \pm 71$ and $208 \pm 49\%$, $P < 0.05$ and $P < 0.01$, respectively), but not in Th1 cells ($110 \pm 41\%$, Figure 3b). Thus, these data show a difference in $PGE_2$ functionality in Th1 cells *versus* Th2 and freshly isolated T cells. Together, our findings suggest that the defective regulation of cytokine production by cAMP elevating in polarized T helper is caused by the inability of $PGE_2$ receptor and/or $\beta_2$-AR stimulation to increase intracellular cAMP levels.

As previously described, one of the mechanisms responsible for the impaired capacity to increase cAMP accumulation may be the upregulation of cAMP-specific PDE's (Seybold *et al.*, 1998), which are responsible for the degradation of cAMP. However, basal levels of cAMP were not reduced in polarized T helper cells compared to freshly isolated T cells, indirectly indicating that basal activity of PDE's is not enhanced. Additionally, the responsiveness of cytokine production to PDE inhibitors was not enhanced in polarized T cells compared to freshly isolated T cells (data not shown). Thus,

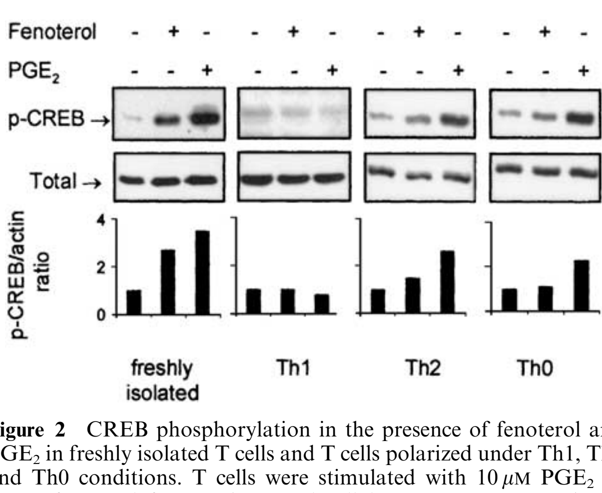


**Figure 2** CREB phosphorylation in the presence of fenoterol and $PGE_2$ in freshly isolated T cells and T cells polarized under Th1, Th2 and Th0 conditions. T cells were stimulated with 10 $\mu$M $PGE_2$ or 10 $\mu$M fenoterol for 60 min. Total cell lysates were prepared and phosphorylated CREB was detected by Western blotting. Phospho-CREB is depicted in the upper panel and total protein levels (actin) are shown in the lower panel (marked by arrows). The corresponding diagram shows the relative phospho-CREB values after normalisation for actin. Results shown are representative of three independent experiments.

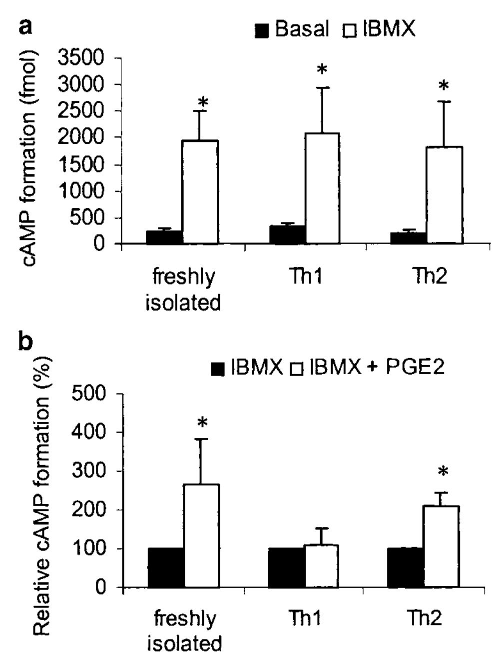


**Figure 3** (a) Incubation with IMBX strongly enhances cAMP accumulation in Th1, Th2 and freshly isolated T cells. Basal intracellular cAMP accumulation (fmol) and cAMP accumulation in the presence of IBMX (100 $\mu$M) was measured in freshly isolated T cells and polarized Th1 and Th2 cells. Values are presented as the means $\pm$ s.e.m. of four independent experiments. $^*P < 0.001$ for the values with IBMX compared to the values without IBMX. (b) $PGE_2$ enhances cAMP formation in freshly isolated T cells and polarized Th2 cells, but not in polarized Th1 cells. cAMP accumulation was induced by $PGE_2$ (10 $\mu$M) in the presence of IBMX and measured in freshly isolated T cells and Th1 and Th2 cells. cAMP levels are expressed as percentage ($x \pm$ s.e.m., $n = 4$) of the formation in absence of $PGE_2$. $^*P < 0.05$ for the values with $PGE_2$ compared to the values without $PGE_2$.

PDE's are unlikely to play a role in the reduced responsiveness to cAMP elevating substances.

### *CREB phosphorylation is induced and cytokine production is strongly inhibited by db-cAMP, IBMX and direct stimulation of the $G_s$ protein in polarized Th1 and Th2 cells*

Next, it was of interest to study whether cAMP is still able to activate its downstream pathway and to negatively regulate cytokine production in polarized T helper cells. As demonstrated in Figure 4a, strong induction of CREB phosphorylation was observed when db-cAMP was added to Th1 cells. These results clearly demonstrate that cAMP-induced signaling is not impaired in these cells. Moreover, addition of IBMX increased both cAMP accumulation (see the results described above) and CREB phosphorylation in Th1 cells, indicating that intracellular cAMP generation is not disturbed. Finally, direct activation of the AC-coupled $G_s$ protein by NaF resulted in strong induction of CREB phosphorylation. This indicates that the defective induction of CREB phosphorylation by $PGE_2$ and fenoterol is most likely caused at receptor level. Similar results with db-cAMP, IBMX and NaF were observed in Th2 cells (Figure 4a) and freshly isolated T cells (data not shown).

In addition, we examined whether IBMX or db-cAMP could also inhibit IFN-$\gamma$ and IL-5 protein secretion in Th1 and Th2 cells, respectively. Indeed, enhancement of intracellular levels of cAMP by the addition of IBMX reduced $\alpha$-CD3/$\alpha$-CD28 stimulated IL-5 and IFN-$\gamma$ protein secretion by approximately 85%. Similar results were obtained with cAMP analog db-cAMP (Figure 4b). These data demonstrate that cytokine production can still be efficiently regulated by the cAMP-dependent pathway in polarized T helper cells, supporting the findings that the reduced responsiveness to $PGE_2$ and/or $\beta_2$-agonist fenoterol in polarized Th cells is because of impaired activation of the AC system and not differential regulation of cytokine production.

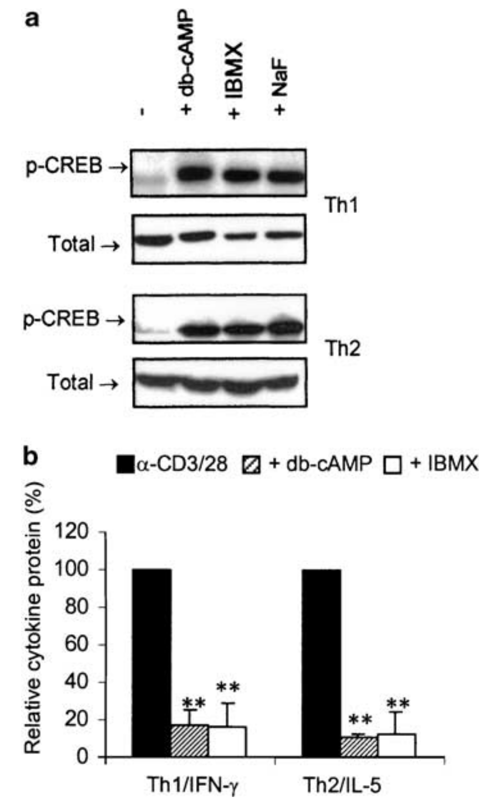


**Figure 4** (a) CREB phosphorylation is induced by cAMP elevating agents in polarized Th1 and Th2 cells. T cell subsets were stimulated with db-cAMP, IBMX or NaF for 60 min. Total cell lysates were prepared and phosphorylated CREB was detected by Western blotting. Phospho-CREB is depicted in the upper panel and total protein levels (actin) are shown in the lower panel (marked by arrows). Results shown are representative of three independent experiments. (b) Cytokine production in polarized Th1 and Th2 cells is under strict control of potent activators of the cAMP-dependent pathway. Polarized Th1 and Th2 cells were cultured overnight in RPMI medium containing 5% FCS and stimulated for 6 h with $\alpha$-CD3/$\alpha$-CD28. IBMX or db-cAMP was added prior to stimulation, in a concentration of 100 $\mu$M, 0.5 and 10 mM respectively. IFN-$\gamma$ and IL-5 protein secretions were measured in cell-free supernatants. Protein levels are expressed as percentage ($x \pm$s.e.m., $n=3$) of the secretion after stimulation with $\alpha$-CD3/$\alpha$-CD28 in the absence of cAMP elevating agents. $^*P<0.05$ for the cytokine secretion level after preincubation with cAMP elevating agents compared to the level without these agents.

### *Enhanced expression of βARK (GRK3) in polarized T helper cells*

We were interested in the possible mechanisms involved in the desensitization of the $PGE_2$ and/or fenoterol effects in polarized Th1 and Th2 cells. Desensitization of the $\beta_2$-AR can be induced by either downregulation of mRNA and protein levels of the receptor (Rademaker *et al.*, 1990) or by phosphorylation of the receptor, which results in functional uncoupling from the $G_s$ protein and internalization of the receptor. First, the expression level of mRNA was determined using RT – PCR. We observed that, similar to freshly isolated T cells, $\beta_2$AR mRNA was clearly expressed in both Th1 and Th2 subsets (Figure 5a). Thus, downregulation of $\beta_2$AR mRNA is not likely the cause of desensitization of the $\beta_2$-adrenergic system in Th1 and Th2 cells. GRK's, as well as PKA and protein kinase C (PKC) are able to phosphorylate the $\beta_2$-AR (Meurs *et al.*, 1987; Hausdorff *et al.*, 1990). Enhanced expression and activation of $\beta$ARK are known to induce $\beta_2$-AR desensitization because of enhanced and more rapid phosphorylation of the receptor (Lohse *et al.*, 1992; McGraw & Liggett, 1997; Penn *et al.*, 1998). It has been described that treatment of T cells with polyclonal activators (PHA, $\alpha$-CD3 and IL-2) for 3 – 7 days results in enhanced $\beta$ARK (GRK3) and GRK6 mRNA and protein levels as well as increased activity of both kinases (Loudon *et al.*, 1996). Therefore, we analyzed the levels of $\beta$ARK2/GRK3 in polarized Th cells and freshly isolated T cells by immunoblotting. As demonstrated in Figure 5b, the expression of $\beta$ARK was dramatically enhanced in polarized Th1 cells compared to freshly isolated T cells. The expression in Th2 cells was lower, but still strongly enhanced compared to freshly isolated T cells. In addition, we studied the expression of $\beta$ARK in T cells cultured under neutral conditions, which also showed defective induction of CREB phosphorylation by fenoterol. In these cells, levels of $\beta$ARK were strongly enhanced compared to freshly isolated T cells as well. Thus, polyclonal activation of T

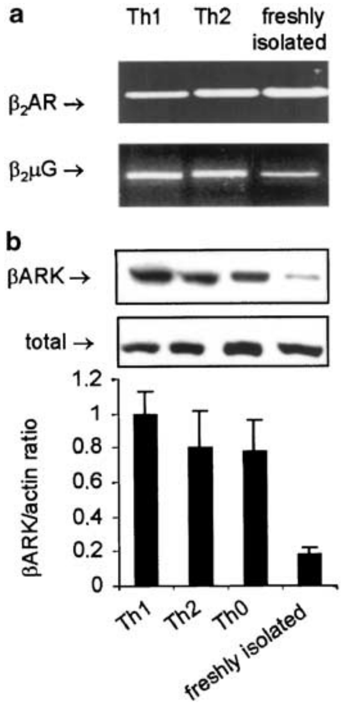


**Figure 5** (a) $\beta_2$AR mRNA is clearly expressed in Th1 and Th2 cells. RNA was isolated from polarized Th1 and Th2 cells and total cellular RNA was analyzed by RT – PCR. In the upper panel, the $\beta_2$AR signal is shown. The $\beta_2\mu$G signal in the lower panel shows that comparable amounts of product were amplified for each condition. Results shown are representative of two independent experiments. (b) Expression of $\beta$ARK is strongly enhanced in polarized Th1, Th2 and Th0 cells compared to freshly isolated T cells ($n = 3$). Expression of $\beta$ARK in polarized Th1 and Th2 cells was analyzed by Western blotting. $\beta$ARK is depicted in the upper panel and actin is depicted in the lower panel. A representative blot is shown. The $\beta$ARK levels in Th1, Th2, Th0 and freshly isolated T cells were normalized for actin and mean relative $\beta$ARK values $\pm$ s.e.m. of four different experiments are depicted in the corresponding diagram.

cells during polarization might induce an increase in $\beta$ARK expression, which in turn might be involved in the loss of $\beta_2$-adrenergic control.

### *Altered levels of PGE$_2$ receptor subtypes in polarized T helper cells*

In addition to $\beta_2$-adrenergic unresponsiveness, polarized Th1 were unresponsive to PGE$_2$. Although, the mechanism of $\beta_2$AR desensitization has been widely described, little information is available about desensitization and internalization of the PGE$_2$ receptor. It is known that PGE$_2$ can exert differential effects through activation of different subtypes of the receptor. At least four different subtypes are known, that is, the EP$_1$, EP$_2$, EP$_3$ and EP$_4$ subtypes. It has been described that EP$_4$ is susceptible to agonist-promoted internalization, whereas the EP$_2$ receptor is resistant. EP$_2$ and EP$_4$ are known to be coupled to the G$_s$ protein and induce AC activation. On the other hand, activation of EP$_1$ induces $Ca^{2+}$ accumulation, while the EP$_3$ subtype preferentially couples to G$_i$, thereby inhibiting cAMP generation (An *et al*., 1993; Funk *et al*., 1993; Yang *et al*., 1994; Breyer & Breyer, 2001; Castleberry *et al*., 2001). To study if differential

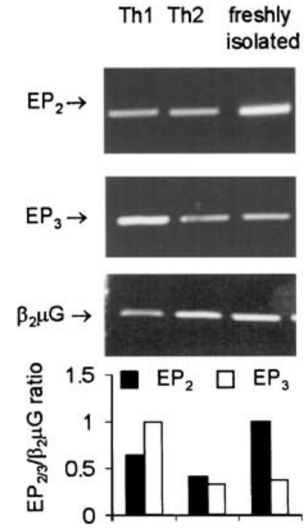


**Figure 6** Altered expression of the EP$_2$ and EP$_3$ subtypes of the PGE$_2$ receptor in Th1 and Th2 cells compared to freshly isolated T cells. RNA was isolated from polarized Th1 and Th2 cells and total cellular RNA was analyzed by RT – PCR. In the upper panel, the EP$_2$ signal shows reduced expression in Th1 and Th2 cells compared to freshly isolated T cells. In the middle panel, the EP$_3$ signal shows enhanced expression in Th1 cells compared to Th2 cells and freshly isolated T cells. The $\beta_2\mu$G signal is depicted in the lower panel. The corresponding diagram shows the relative EP$_2$ and EP$_3$ mRNA values after normalization for $\beta_2\mu$G. Results shown are representative of three independent experiments.

expression of the PGE$_2$ receptor could be involved in the altered PGE$_2$ signaling in polarized T helper cells, we measured the mRNA expression of the G$_s$-coupled EP$_2$ subtype and the G$_i$-coupled EP$_3$ subtype of the PGE$_2$ receptor using RT – PCR. As demonstrated in Figure 6, the expression of EP$_2$ was reduced in both Th1 and Th2 cells compared to freshly isolated T cells. This may be involved in the reduced responsiveness of cytokine production to PGE$_2$ that was observed in both Th1 and Th2 cells compared to freshly isolated T cells; yet, this does not explain the difference of PGE$_2$ reactivity in Th1 and Th2 cells. As described above, PGE$_2$ was able to activate the AC/cAMP-dependent pathway in Th2 cells, while this was not the case in Th1 cells. In contrast to the G$_s$-coupled EP$_2$, subtype, the expression of the G$_i$-coupled EP$_3$ subtype appeared to be about a two-fold higher in Th1 cells compared to freshly isolated T cells as well as Th2 cells (Figure 6). Since activation of the G$_s$ protein is known to result in activation of the AC/cAMP system, while activation of the G$_i$ protein is known to inhibit this system, the altered ratio in EP$_2$ and EP$_3$ expression in Th1 cells might be an explanation for the net zero effect of PGE$_2$ on cAMP production in Th1 cells (see Figure 3b). Thus, differential expression of subtypes of the PGE$_2$ receptor might be involved in the unresponsiveness of Th1 cells to PGE$_2$.

## Discussion

Specialized Th1 and Th2 subsets direct immune responses at sites of inflammation by the production of a defined pattern of cytokines. An important regulator of proinflammatory activity is the cAMP-dependent pathway. At sites of tissue inflammation, T cells have ample opportunity to encounter cells that produce cAMP elevating agents, for example, $PGE_2$. The regulatory effects of cAMP have been widely studied in freshly isolated T cells and in T-cell clones, but not in specialized T helper subset cells, which can be obtained by *in vitro* differentiation under Th1 or Th2 polarizing conditions. In the present report, we demonstrate that cytokine secretion in polarized T helper cells is not strictly controlled by cAMP elevating agents. Although the α-CD3/α-CD28 stimulation method cannot necessarily be extrapolated to the activation of T cells by antigen, our results may have important implications for the regulation of Th1- and Th2-mediated immune responses. Our findings are in contrast to the results in freshly isolated T cells, where the production of the Th1-like cytokine IFN-$\gamma$ was almost fully blocked by cAMP elevating substances and significant inhibition of Th2-like cytokine (IL-4, IL-5) production was observed. The inhibitory effect on IFN-$\gamma$ secretion appeared to be completely abolished in polarized Th1 cells, while the inhibitory effect on IL-4 and IL-5 in polarized Th2 cells was reduced compared to freshly isolated T cells. Thus, whereas in freshly isolated T cells, Th1 cytokine production is more susceptible to cAMP inhibition than Th2 cytokine production, polarized Th2 cells appear to be more sensitive to $PGE_2$ than polarized Th1 cells. $PGE_2$ was able to induce an increase in cAMP production and CREB phosphorylation in Th2 cells, while $PGE_2$ was unable to enhance cAMP production and subsequently activate downstream signals in Th1 cells. This may have implications for the Th1/Th2 balance at sites of tissue inflammation, where specialized Th1 or Th2 subsets may be found. Our data indicate that the loss of $PGE_2$ control over IFN-$\gamma$ production in polarized Th1 cells is most likely because of a defect at receptor level and is not caused by differential regulation of cytokine production. This is supported by the finding that preincubation with db-cAMP, IBMX or NaF significantly enhances CREB phosphorylation and strongly inhibits IFN-$\gamma$ production, indicating that IFN-$\gamma$ production is still under the control of the cAMP-dependent pathway. Defective induction of CREB phosphorylation might indeed lead to a loss of control over IFN-$\gamma$ production, since it has been shown that CREB inhibits Jun-mediated activation of the IFN-$\gamma$ promoter by competitive binding (Zhang *et al*., 1998). In addition to the regulation of cytokine production, the cAMP-dependent pathway has been described to induce apoptosis in T lymphocytes (Gu *et al*., 2000). Thus, our results might also have implications for the survival of Th1 and Th2 subsets in inflamed tissue, where T cells have opportunity to encounter $PGE_2$ secreting cells.

In contrast to the differential effects of $PGE_2$ in Th1 and Th2 subsets, we found reduced responsiveness to the $\beta_2$-agonist fenoterol in both Th1 and Th2 cells. This seems to be because of a defect at receptor level, since the ability of fenoterol to activate the cAMP-dependent pathway was impaired in polarized T cells. If the *in vitro* polarized T cells are indeed representative for specialized Th1 and Th2 subsets *in vivo*, our results suggest that the use of $\beta_2$-mimetics, for instance in asthma, may not efficiently inhibit proinflammatory T-cell activity. Several mechanisms may be responsible for the desensitization of the $\beta_2$-AR. First, prolonged agonist binding can result in downregulation of the total cellular levels of the receptor (both mRNA and protein) and contribute to desensitization of the receptors (Rademaker *et al*., 1990). However, no indications for a prolonged repression of receptor gene transcription, resulting in decreased receptor expression, were observed in polarized Th cells; mRNA for the $\beta_2$AR was clearly expressed in polarized Th1 cells and Th2 cells. Thus, a functional uncoupling of the $\beta_2$AR and $PGE_2$ receptor from the $G_s$ protein seems to be more likely. This can be caused by phosphorylation of the receptor by GRKs, PKA or PKC (Meurs *et al*., 1987; Hausdorff *et al*., 1990). Phosphorylation of the active form of the receptor by $\beta$ARK (also GRK3) promotes binding of $\beta$-arrestin to the receptor, resulting in uncoupling from the $G_s$ protein and finally internalization of the receptor (Gu *et al*., 2000). Enhanced expression and activation of $\beta$ARK may induce $\beta_2$-adrenergic hyporesponsiveness (Lohse *et al*., 1992). It has been demonstrated that overexpression of $\beta$ARK leads to hyporesponsiveness of the $\beta_2$-AR because of enhanced and more rapid agonist-induced phosphorylation of the receptor (McGraw & Liggett, 1997; Penn *et al*., 1998). Indeed, we found that $\beta$ARK levels in polarized Th1 and Th2 cells were strongly enhanced compared to freshly isolated T cells. In addition, high levels of $\beta$ARK were observed in T cells polarized under neutral (Th0) conditions, where reduced responsiveness to $\beta_2$-agonist fenoterol was also found. These data suggest that polyclonal activation of T cells during polarization induces upregulation of $\beta$ARK, thereby reducing the responsiveness to $\beta_2$-agonists. In this way, T cells may become less sensitive to circulating $\beta_2$-agonists (e.g. epinephrine) during the induction of cell cycle progression at the initiation of immune responses.

So far, little information is available on the regulation of the $PGE_2$ receptor. It has been demonstrated that the $EP_4$ subtype of the $PGE_2$ receptor is susceptible to agonist-promoted internalization, whereas the $EP_2$ subtype receptor is resistant (Penn *et al*., 2001). $EP_4$ desensitization can occur by a similar mechanism as $\beta_2$-adrenergic desensitization, involving $\beta$ARK and $\beta$-arrestin. Therefore, a role for enhanced $\beta$ARK expression in $PGE_2$ unresponsiveness in Th1 cells cannot be excluded, although it is not clear why Th2 cells, expressing high levels of $\beta$ARK, were still responsive to $PGE_2$. We show that another possible mechanism involved in reduced responsiveness to $PGE_2$ might be the altered balance in $EP_2/EP_3$ mRNA expression. $EP_2$ mRNA was reduced in both Th1 and Th2 subsets compared to freshly isolated T cells, whereas $EP_3$ mRNA expression was only enhanced in Th1 cells. Thus, the reduced expression of $EP_2$ could explain why Th2 cells are less responsive to $PGE_2$ than freshly isolated T cells, while the additional upregulation of mRNA for the $G_i$-coupled $EP_3$ receptor in Th1 cells may be responsible for the complete unresponsiveness to $PGE_2$ in these cells. It is known that activation of the $G_i$ protein by $PGE_2$ and $\beta_2$-agonists does not result in cAMP formation, but instead causes activation of the PI3-kinase-dependent antiapoptotic pathways (Zhu *et al*., 2001). Our data suggest that coupling of the $PGE_2$ receptors to the $G_i$ protein is enhanced in polarized Th1 cells compared to freshly isolated T cells. This might result in a net zero effect on cAMP production and activation of downstream pathways, since activation of $G_i$ counteracts the effects induced by activation of the $G_s$ protein. It still remains unclear as to what causes the Th1 specific upregulation of $EP_3$ mRNA. Incubation of T cells with the Th1-directing cytokine IL-12 alone for prolonged periods did not result in reduced ability of $PGE_2$ to activate the cAMP-dependent pathway (data not shown). Possibly, entering the cell cycle and epigenetic remodeling during Th1 polarization are required to induce upregulation of

$EP_3$ expression. Another G-coupled receptor that can trigger different intracellular events through different receptor subtypes is the histamine receptor. It has been demonstrated that histamine reduces cytokine (IL-4, IL-13) production and elevates cAMP production in differentiated murine Th2 cells, which preferentially expressed the histamine receptor type 2 (HR2) (Jutel *et al*., 2001). In contrast, Th1 cells preferentially expressed HR1, leading to an increase in calcium influx instead of an increase in intracellular cAMP levels upon activation and in addition an upregulation of IFN-$\gamma$ production (Jutel *et al*., 2001). Thus, reduced linking of G protein-coupled receptors to the AC system seems to be a general phenomenon in polarized Th1 cells. Together, our findings suggest that resting T cells in peripheral blood are under strict control of $G_s$ protein-coupled receptors, while this control is lost when T cells become activated at the sites of tissue inflammation and differentiate into effector T cells. The loss of control may allow appropriate activation and increase the survival of effector Th cells. Th1 cells appear to be less responsive to G protein-coupled receptors than Th2 cells, favoring activation of the Th1 subtype.

In summary, our study demonstrates that polarized T helper cells are less responsive to $PGE_2$ and the $\beta_2$-agonist fenoterol than circulating T cells. This lack of negative feedback control may have implications for ongoing inflammatory processes, since cytokine production in effector Th2 cells may not be efficiently suppressed and the activity of Th1 cells may not be suppressed at all by a $\beta_2$-agonist or $PGE_2$ secreted by surrounding tissue cells.

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## References


ABBAS, A., MURPHY, K.M. & SHER, A. (1996). Functional diversity of helper T lymphocytes. *Nature,* **383,** 787 – 793.

AN, S., YANG, J., XIA, M. & GOETZL, E.J. (1993). Cloning and expression of the EP2 subtype of human receptors for prostaglandin E2. *Biochem. Biophys. Res. Commun.,* **197,** 263 – 270.

ARGAWAL, S. & RAO, A. (1998). Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity,* **9,** 765.

BETZ, M. & FOX, B.S. (1991). Prostaglandin E2 inhibits the production of Th1 lymphokines but not of Th2 cytokines. *J. Immunol,* **146,** 108 – 113.

BIRD, J.J., BROWN, D.R., MULLEN, A.C., MOSKOWITZ, N.H., MAHOWALD, M.A., SIDER, J.R., GAJEWSKI, T.F., WANG, & C.R. & REINER, S.L. (1998). Helper T cell differentiation is controlled by the cell cycle. *Immunity,* **9,** 229.

BREYER, M.D. & BREYER, R.M. (2001). G protein-coupled prostanoid receptors and the kidney. *Annu. Rev. Physiol.,* **63,** 579 – 605.

BORGER, P., KAUFFMAN, H.F., POSTMA, D.S., VELLENGA, E. (1996). Interleukin-4 gene expression in activated human T lymphocytes is regulated by the cyclic adenosine monophosphate-dependent signaling pathway. *Blood,* **87,** 691 – 698.

BORGER, P., TEN HACKEN, N.T.H., VELLENGA, E., KAUFFMAN, H.F. & POSTMA, D.S. (1999). Peripheral blood T lymphocytes from asthmatic patients are primed for enhanced expression of interleukin (IL)-4 and IL-5 mRNA: association with lung function and serum IgE. *Clin. Exp. Allergy,* **29,** 772 – 779.

BORGER, P., VELLENGA, E., GRINGHUIS, S.I., TIMMERMAN, A.B., LUMMEN, C., POSTMA, D.S. & KAUFFMAN, H.F. (1998). Prostaglandin $E_2$ differently modulates interleukin-5 gene expression in activated human T lymphocytes depending on the costimulatory signal. *J. Allergy Clin. Immunol.,* **101,** 231 – 240.

CASTLEBERRY, T.A., LU, B., SMOCK, S.L. & OWEN, T.A. (2001). Molecular cloning and functional characterization of the canine prostaglandin E2 receptor EP4 subtype. *Prostaglandins Other Lipid Medial.,* **65,** 167 – 187.

FUNK, C.D., FURCI, L., FITZGERALD, G.A., GRYGORCZYK, R., ROCHETTE, C., BAYNE, M.A., ABRAMOVITZ, M., ADAM, M. & METTIERS, K.M. (1993). Cloning and expression of a cDNA for the human prostaglandin E receptor EP1 subtype. *J. Biol. Chem.,* **268,** 26767 – 26772.

GONZALEZ, G.A. & MONTMINY, M.R. (1889). cAMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell,* **59,** 675 – 680.

GU, C., MA, Y.C., BENJAMIN, J., LITTMAN, D., CHAO, M.V. & HUANG, X.Y. (2000). Apoptotic signaling through the $\beta$-adrenergic receptor. A new $G_s$ effector pathway. *J. Biol. Chem.,* **275,** 20726 – 20733.

HARADA, Y., MIYATAKE, S., ARAI, K. & WATANABE, S. (1999). Cyclic AMP inhibits the activity of c-Jun N-terminal kinase (JNKp46) but not JNKp55 and ERK2 in human helper T lymphocytes. *Biochem. Biophys. Res. Commun.,* **266,** 129 – 134.

HAUSDORFF, W.P., LOHSE, M.J., BOUVIER, M., LIGGETT, S.B., CARON, M.G., LEFKOWITZ, R.J. (1990). Two kinases mediate agonist-dependent phosphorylation and desensitization of the beta 2-adrenergic receptor. *Symp. Soc. Exp. Biol.,* **44,** 225 – 240.

HILKENS, C.M., VERMEULEN, U.M.H., VAN NEERVEN, R.J.J., SNIJDEWINT, F.G.M., WIERENGA, E.A. & KAPSENBERG, M.L. (1995). Differental modulation of T helper type 1 (Th1) and T helper type 2 (Th2) cytokine secretion by prostaglandin E2 critically depends on interleukin-2. *Eur. J. Immunol.,* **25,** 59 – 63.

HOEKSTRA, M.O., HOEKSTRA, Y., DE REUS, D., RUTGERS, B., GERRITSEN, J. & KAUFFMAN, H.F. (1997). Interleukin-4 (IL-4) and interferon-$\gamma$ and interleukin-5 (IL-5) in peripheral blood of children with moderate atopic asthma. *Clin. Exp. Allergy,* **27,** 1254 – 1260.

HSIEH, C.S., MACATONIA, S.E., TRIPP, C.S., WOLF, S.F., O GARRA, A. & MURPHY, K.M. (1993). Development of TH1 CD4 + T cells through IL-12 produced by Listeria-induced macrophages. *Science,* **260,** 547 – 549.

JUNG, T., SCHAUER, U., HEUSSER, C., NEUMANN, C. & RIEGER, C. (1993). Detection of intracellular cytokines by flow cytometry. *J. Immunol. Methods,* **159,** 197 – 207.

JUTEL, M., WATANABE, T., KLUNKER, S., AKDIS, M., THOMET, O.A., MALOLEPSZY, J., ZAK-NEJMARK, T., KOGA, R., KOBAYASHI, T., BLASER, K. & AKDIS, C.A. (2001). Histamine regulates T-cell and antibody responses by differential expression of H1 and H2 receptors. *Nature,* **413,** 420 – 425.

LEE, H.J., MATSUDA, I., NAITO, Y., YOKOTA, T., ARAI, N. & ARAI, K. (1994). Signals and nuclear factors that regulate the expression of interleukin-4 and interleukin-5 genes in helper T cells. *J. Allergy Clin. Immunol.,* **94,** 594 – 604T.

LOHSE, M.J., ANDEXINGER, S., PITCHER, J., TRUKAWINSK, I.S., CODINA, J., FAURE, J.P., CARON, M.G. & LEFKOWITZ, R.J. (1992). Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. *J. Biol. Chem.,* **267,** 8558 – 8564.

LOUDON, R.P., PERUSSIA, B. & BENOVIC, J.L. (1996). Differentially regulated expression of the G-protein-coupled receptor kinases, beta-ARK and GRK6, during myelomonocytic cell development *in vitro*. *Blood,* **88,** 4547 – 4557.

MASQUILIER, D. & SASSONE-CORSI, P. (1992). Transcriptional cross-talk: nuclear factors CREM and CREB bind to AP-1 sites and inhibit activation by Jun. *J. Biol. Chem.,* **267,** 22460 – 22466.

MCGRAW, D.W. & LIGGETT, S.B. (1997). Heterogenity in $\beta_2$-adrenergic receptor kinase expression in the lung accounts for

cell-specific desensitization of the $\beta_2$-adrenergic receptor. *J. Biol. Chem.,* **272,** 7338 – 7344.
MEURS, H., KAUFFMAN, H.F., KOETER, G.H. & DE VRIES, K. (1980). Extraction of cyclic AMP for the determination in the competitive protein binding assay. *Clin. Chim. Acta,* **106,** 91 – 97.
MEURS, H., KAUFFMAN, H.F., KOETER, G.H., TIMMERMANS, A. & DE VRIES, K. (1987). Regulation of the beta-receptor-adenylate cyclase system in lymphocytes of allergic patients with asthma: possible role for protein kinase C in allergen-induced nonspecific refractoriness of adenylate cyclase. *J. Allergy Clin. Immunol.,* **80,** 326 – 339.
NIGG, E.A., HILZ, H., EPPENBERGER, H.M. & DUTLY, F. (1985). Rapid and reversible translocation of the catalytic subunit of cAMP-dependent protein kinase type II from the Golgi complex to the nucleus. *EMBO J.,* **4,** 2801 – 2806.
O'GARRA, A. (1998). Cytokines induce the development of functionally heterogenous T helper subsets. *Immunity,* **8,** 275 – 283.
PENN, R.B., PASCUAL, R.M., KIM, Y.M., MUNDELL, S.J., KRYMSKAYA, V.P., PANETTIERI, R.A. & BENOVIC, J.L. (2001). Arrestin specificity for G protein-coupled receptors in human airway smooth muscle. *J. Biol. Chem.,* **276,** 32648 – 32656.
PENN, R.B., REYNOLD, A., PANETTIERI, J.R. & BENOVIC, J.L. (1998). Mechanisms of acute desensitization of the $\beta_2$AR-adenylyl cyclase pathway in human airway smooth muscle. *Am. J. Resp. Cell Mol. Biol.,* **19,** 338 – 348.
RADEMAKER, B., KRAMER, K., BAST, A. & TIMMERMAN, H. (1990). Evidence for cell type dependent mechanisms in agonist-induced down-regulation of $\beta$-adrenoceptors. *Res. Commun. Chem. Parthol. Pharmacol.,* **12,** 321 – 336.
ROOZENDAAL, R., VELLENGA, E., DE JONG, M.A., TRAANBERG, K.F., POSTMA, D.S., DE MONCHY, J.G.R. & KAUFFMAN, H.F. (2001). Resistance of activated human Th2 cells to NO-induced apoptosis is mediated by gamma-glutamyltranspeptidase. *Int. Immunol.,* **13,** 519 – 528.
SEDER, R.A., GAZZINELLI, R., SHER, A. & PAUL, W.E. (1993). Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 10188 – 10192.
SEDER, R.A., PAUL, W.E., DAVIS, M.M. FAZEKAS DE ST GROTH, B. (1992). The presence of IL-4 during *in vitro* priming determines the lymphokine-producing potential of CD4+T cells from T cell receptor transgenic mice. *J. Exp. Med.,* **176,** 1091 – 1098.
SEYBOLD, J., NEWTON, R., WRIGHT, L., FINNEY, P.A., SUTTORP, N., BARNES, P.J., ADCOCK, I.M. & GIEMBYCZ, M.A. (1998). Induction of phosphodiesterases 3B, 4A4, 4D1, 4D2, and 4D3 in Jurkat T-cells and in human peripheral blood T-lymphocytes by 8-bromo-cAMP and Gs-coupled receptor agonists. Potential role in beta2-adrenoreceptor desensitization. *J. Biol. Chem.,* **273,** 20575 – 20583.
SNIJDEWINT, F.G.M., KALINSKI, P., WIERTNGA, E.A., BOS, J.D. & KAPSENBERG, M.L. (1993). Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J. Immunol.,* **150,** 5321 – 5329.
TAMIR, A., GRANOT, Y. & ISAKOV, N. (1996). Inhibition of T lymphocyte activation by cAMP is associated with down-regulation of two parallel mitogen-activated protein kinase pathways, the extracellular signal-related kinase and c-Jun N-terminal kinase. *J. Immunol.,* **157,** 1514 – 1522.
WU, J., DENT, P., JELINEK, T., WOLFMAN, A., WBER, M.J. & STURGILL, T.W. (1993). Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine-3′,5′-monophosphate. *Science,* **262,** 1065 – 1069.
YANG, J., XIA, M., GOETZL, E.J. & AN, S. (1994). Cloning and expression of the EP3-subtype of human receptors for prostaglandin E2. *Biochem. Biophys. Res. Commun.,* **198,** 999 – 1006.
ZHANG, F., WANG, D.Z., BOOTHBY, M., PENIX, L., FLAVELL, R.A. & AUNE, T.M. (1998). Regulation of the activity of IFN-$\gamma$ promoter elements during Th cell differentiation. *J. Immunol.,* **161,** 6105 – 6112.
ZHU, W., ZHENG, M., KOCH, W.J., LEFKOWITZ, R.J., KOBILKA, B.K. & XIAO, R. (2001). Dual modulation of cell survival and cell death by $\beta_2$-adrenergic signaling in adult mouse cardiac myocytes. *Proc. Natl. Acad. Sci.,* **98,** 1607 – 1612.

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